Ecoimmunology

- Ecoimmunology is the field of study that attempts to understand the functions of the immune system in the context of the environment of the host.
- Part 1, I will describe how the chytrid pathogen of amphibians evades host immunity.
- Part 2, I will talk about the role of the skin microbiome in protection of newly metamorphosing frogs exposed to the chytrid pathogen.

Ecoimmunology

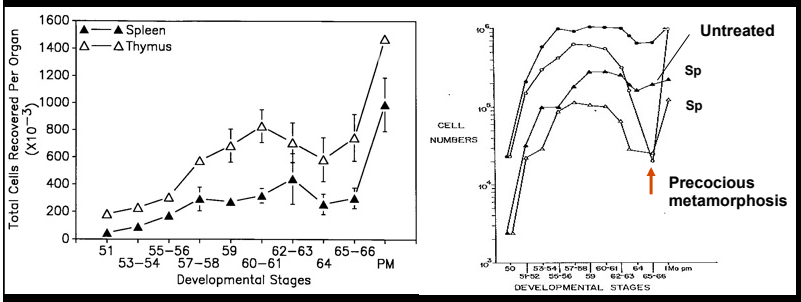
- There are costs and trade-offs necessary to maintain an effective immune defense.
- It is costly in terms of energy to maintain lymphocyte populations if they are not needed.
- Costs may include reduced growth, longevity, or fecundity.
- Trading increased body growth for development of the lymphocyte repertoire may leave the host vulnerable to infection and resulting pathology.

Tradeoffs at metamorphosis

- **Metamorphosis is a unique time for amphibians when many organ systems, including the skin and immune system, change dramatically.**
- **There may be a trade-off between the need for additional energy and immune defense at metamorphosis resulting in the possibility of increased disease.**

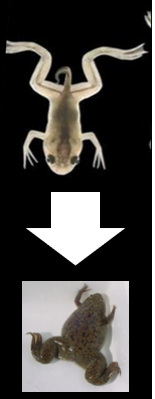
Development of the immune system

- Amphibians have two immune systems, larval and adult
- The larval amphibian immune system is reorganized at metamorphosis.



Redrawn from Rollins-Smith, L.A. et al. 1984. Immunology 52: 491-500;

From Rollins-Smith, L.A. et al. 1988. Differentiation 37:180-185

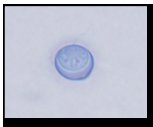


Amphibian declines

- **As of September 21, 2015, there were 7,450 recorded amphibian species (amphibiaweb.org), and new ones are being discovered each year.**
- **Of these, approximately 32 % are declining or at risk for decline** (IUCN Red list)

Panama

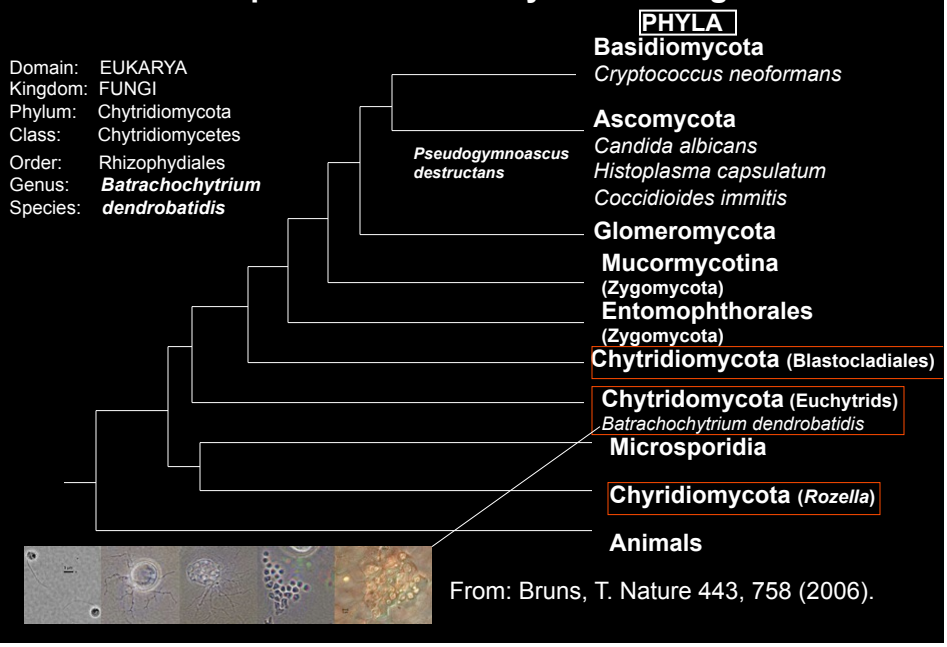
Peru



Amphibian Declines

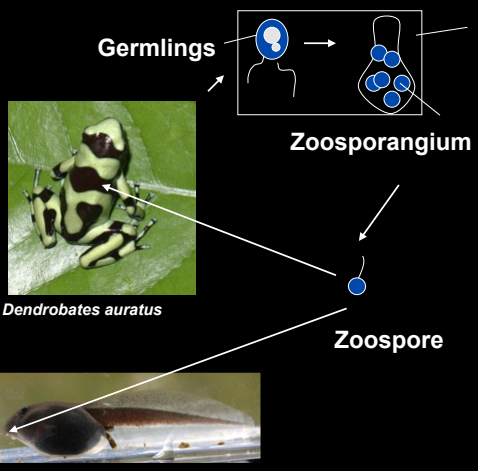
- The causes are complex including habitat destruction, environmental chemicals, and disease.
- My lab has focused our research on disease caused by *Batrachochytrium dendrobatidis (Bd)*, a chytrid fungal pathogen that causes the skin disease chytridiomycosis.
- Until 2013, *Bd* was the only known chytrid pathogenic to vertebrates. With the discovery of *Batrachochytrium salmandrivorans* (*Bsal*), North American salamanders are at increased risk.
  - Thought to have originated in Asia
  - Lethal to European salamanders

Simplified evolutionary tree of fungi

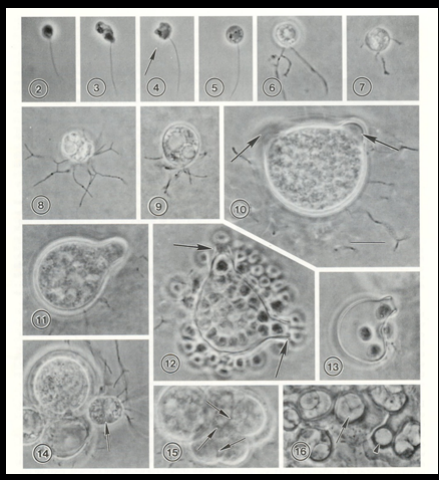


From: Bruns, T. Nature 443, 758 (2006).

Life Cycle of Batrachochytrium dendrobatidis (Bd)



[Stages that occur within frog skin cells]



Duration of life cycle is 4-5 days at 22ºC

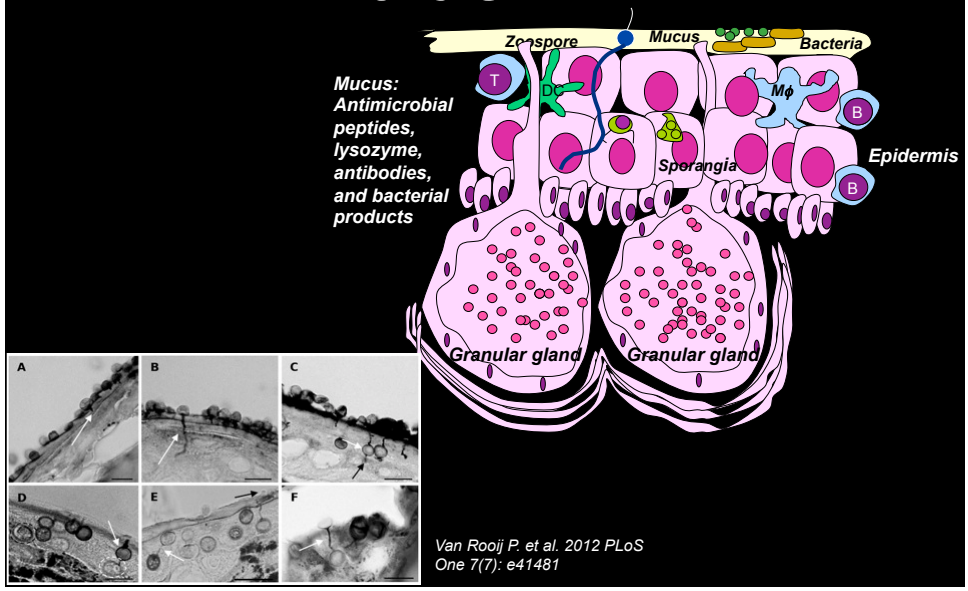
J.E. Longcore et al. 1999. *Mycologia* 91:219

Bd appears to kill by disturbance of skin functions

- Electrolyte transport across ventral skin is impaired in diseased frogs.
- Plasma sodium and potassium concentrations are significantly reduced.

Voyles et al. 2009. *Science* ***326: 582-585.***

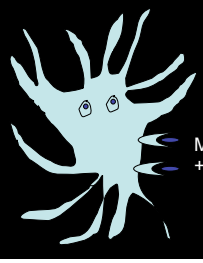
Model of immune defenses in the skin

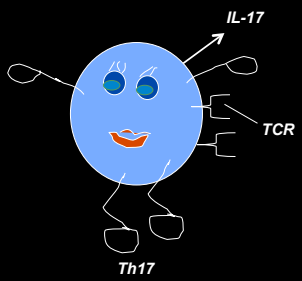


Van Rooij P. et al. 2012 PLoS One 7(7): e41481

How does Bd escape immune surveillance?

- Successful immunity against fungal pathogens begins with recognition by phagocytic cells, which begin to control the infection.
- The phagocytic cells then recruit lymphocyte effectors.
- The lymphocytes amplify the response and recruit more phagocytic cells to clear the infection.

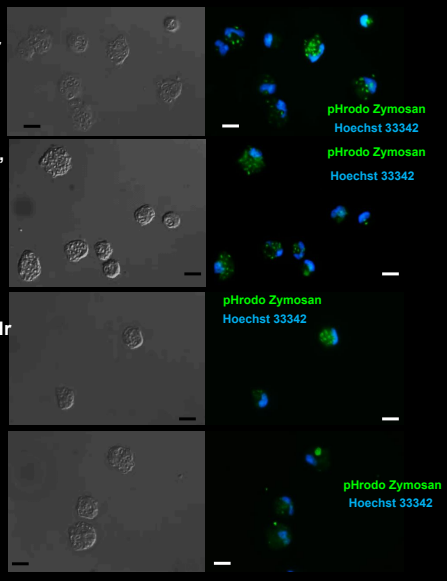




Macrophages and neutrophils can recognize and engulf Bd, and Bd culture supernatants don't impair processing of zymosan into an acidic compartment

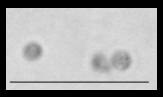
Frog neutrophil has engulfed *Bd* cells

Frog macrophage has engulfed *Bd* cells

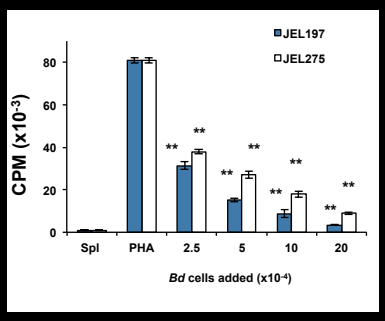


Fites, J.S. et al.** 2013. **Science 342:366-369.

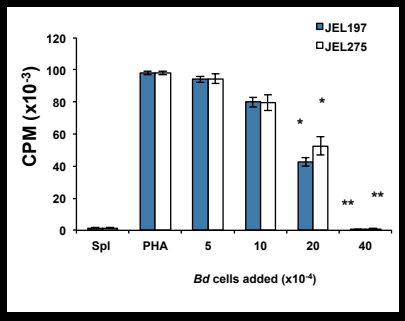
Live or heat killed Bd cells inhibit T lymphocyte proliferation



Live *Bd* co-cultured *with* T cells



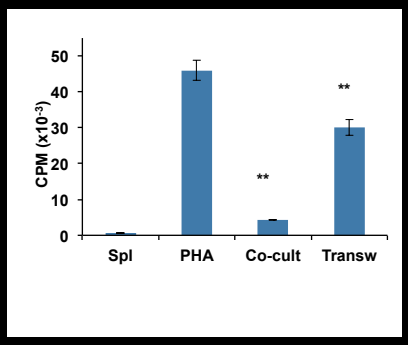
Killed *Bd* co-cultured with T cells

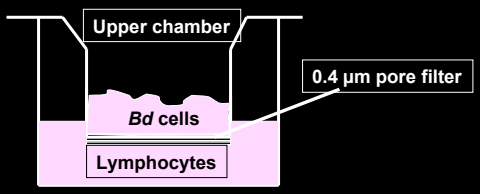


A nonpathogenic chytrid *Homolaphlyctis polyrhiza* does not inhibit lymphocyte proliferation.

Fites, J.S. et al. 2013. Science 342:366-369.

Inhibition of lymphocyte proliferation occurs even when Bd cells are separated from lymphocytes by a 0.4 µm membrane



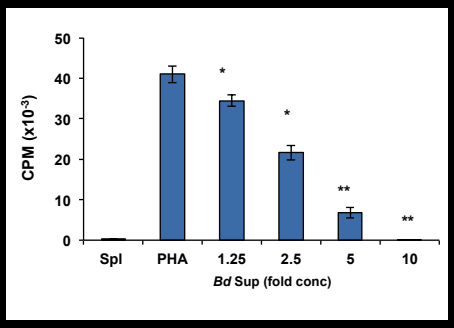


Fites, J.S. et al. 2013. Science 342:366-369.

The inhibitory factors are released by mature sporangia cultured 24 hours in distilled water

10^7 matured *Bd* cells/ml

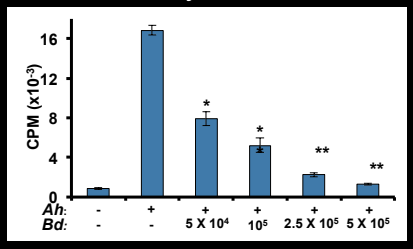
Bd + Glass distilled water



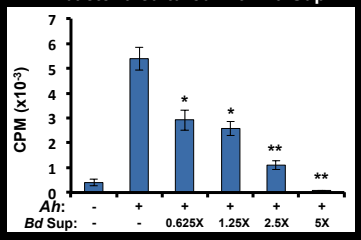
Spin out cells, filter
Lyophilize

The lymphotoxic factors also inhibit B lymphocytes

***Bd* co-cultured with B cells activated by killed bacteria**



B cells activated by killed bacteria cultured with *Bd* Sup

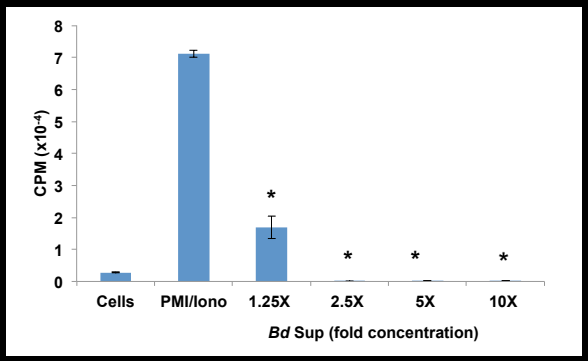


B cell T cell

Therefore, the inhibitory factors target a vulnerability shared by B and T cells.

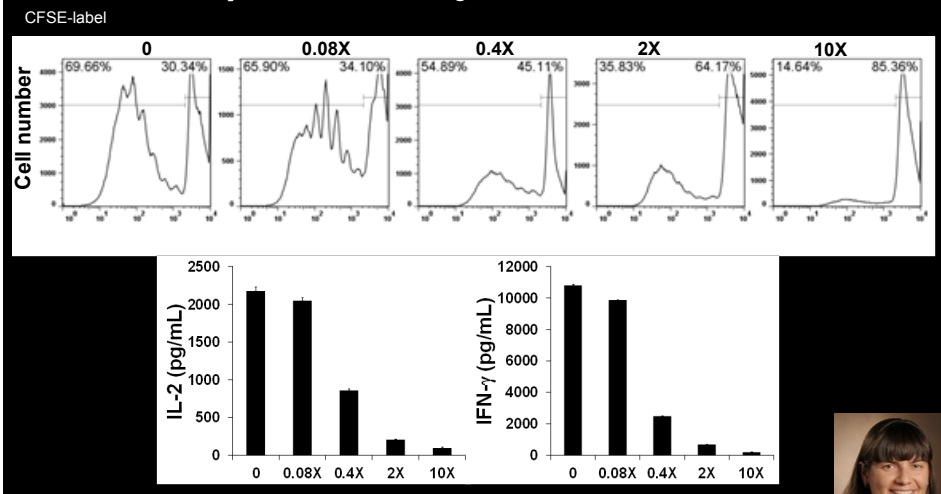
Fites, J.S. et al. 2013. Science 342:366-369.

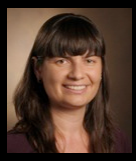
Bd-induced inhibition of lymphocytes is not limited to frogs



With Sarah Parker Collier and Tom Aune

Bd factors also inhibit proliferation and cytokine production by human T cells

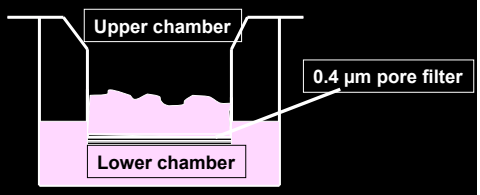


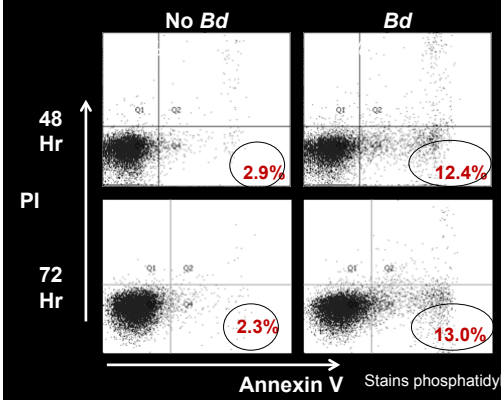


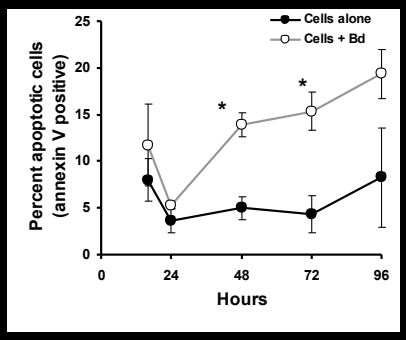
Proliferation of human T cells stimulated with anti-CD3 and anti-CD28 was impaired by *Bd* supernatants. Supernatants from *Bd* cultures inhibited secretion of IL-2 and IFN-γ by purified human CD4+ T cells

With Kyra Richter

One mechanism of inhibition is the induction of lymphocyte apoptosis by Bd cells

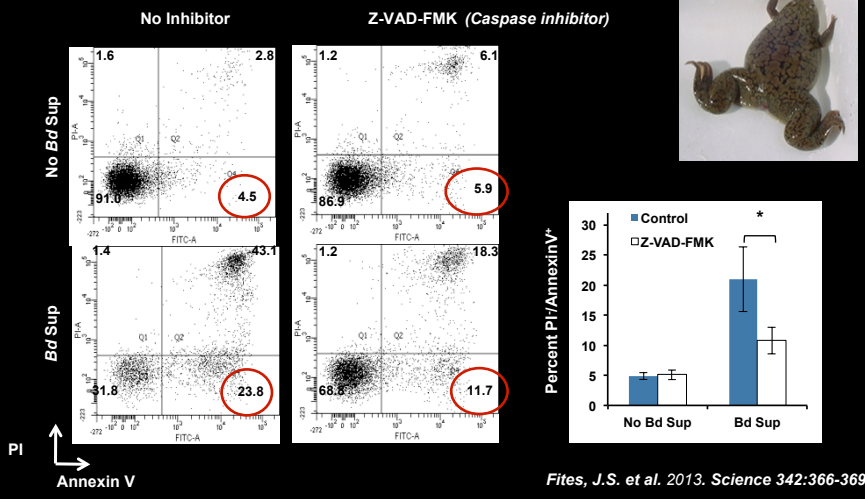






Fites, J.S. et al. 2013. Science 342:366-369.

Lymphocyte apoptosis induced by Bd supernatant is diminished in the presence of a pan caspase inhibitor



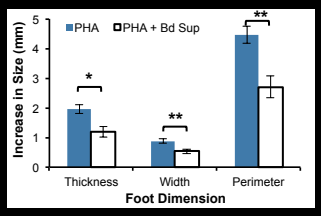
Fites, J.S. et al. 2013. Science 342:366-369.

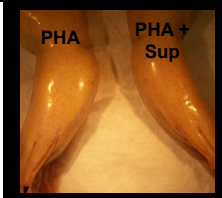
Bd supernatants inhibit a DTH-type response induced by injection of PHA

Day 0 peritoneal injection of PHA (1 mg/ml)

Day 7 injection of PHA (2 mg/ml) into foot with or without *Bd* Sup.

Day 8 and 9 measure foot dimensions.



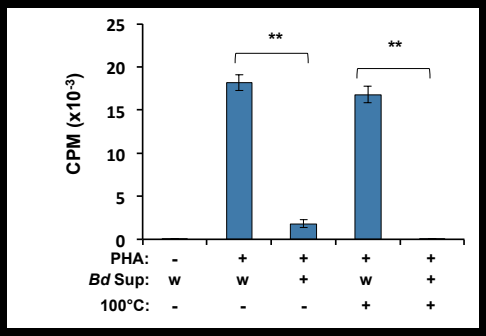


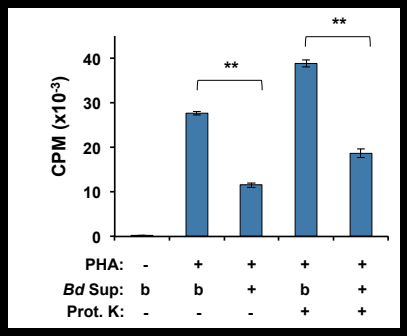
Therefore, *Bd* inhibitory factors can also inhibit a local lymphocyte-mediated response.

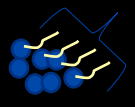
Fites, J.S. et al. 2014 Infect. Immun. 82: 4698-4706.

What is the nature of the inhibitory factors?

***Bd* supernatants retained their capacity to inhibit lymphocyte proliferation following treatment with high heat or proteinase K**





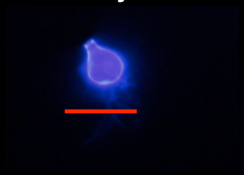


Proteinase K

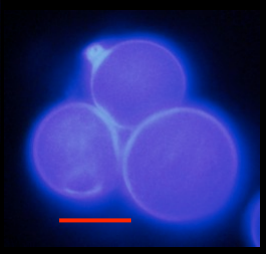
Fites, J.S. et al. 2013. Science ***342:366-369.***

Production of the Bd factors is impaired by treatment with agents that inhibit cell wall synthesis

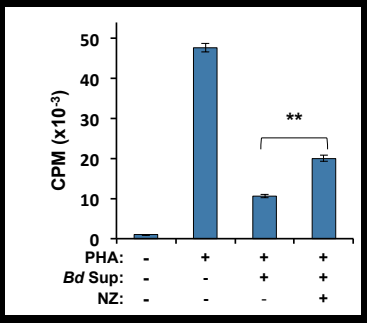
No nikkomycin Z



3.13 µg/ml nikkomycin Z



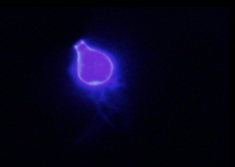
Supernatants from nikkomycin Z-treated cells

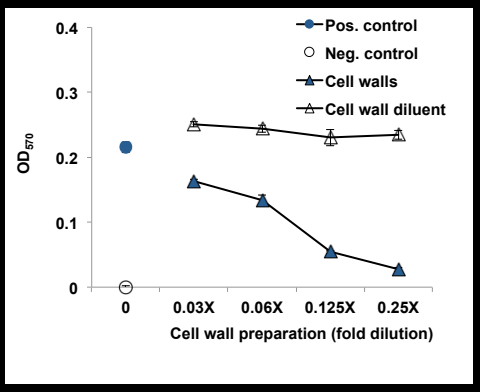


Therefore, the inhibitory factors may be components of the cell wall.

Fites, J.S. et al. 2013. Science 342:366-369.

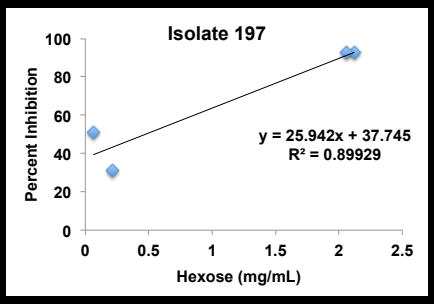
Isolated cell walls have significant inhibitory activity against T cells

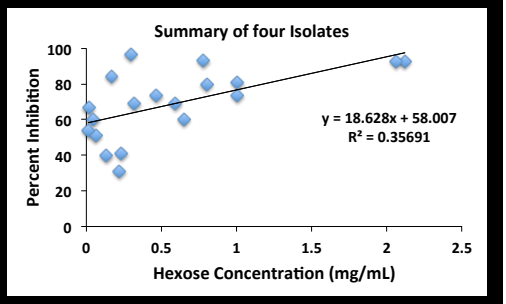




Therefore, some inhibitory factors are enriched in the cell wall.

Inhibition of T cells is positively correlated with carbohydrate concentration of supernatants

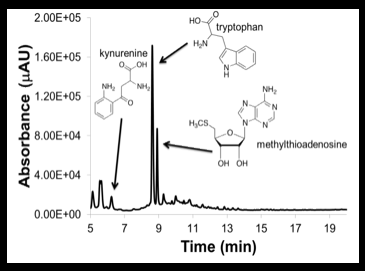


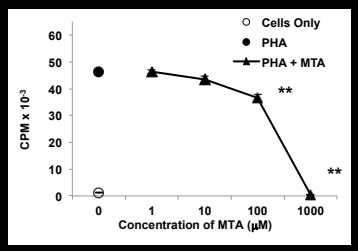


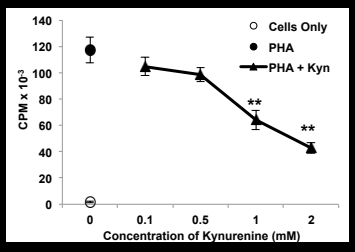
two-tailed t-test, p = 0.0069.

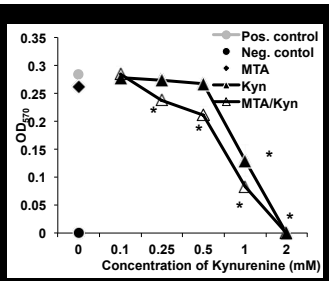
Therefore, the some inhibitory factors may be soluble carbohydrates associated with the cell wall.

HPLC analysis of cell-free Bd supernatants show the presence of tryptophan, kynurenine, and MTA (methylthioadenosine)










Kevin Minbiol


Thomas Umile

Summary of immune evasion by Bd

- ***Bd*** **factors inhibit T and B cells by induction of apoptosis.**
- **The inhibitory factors are water soluble and can cross a cell-impermeable barrier.**
- **The inhibitory factors are heat-resistant and protease-resistant, suggesting that they are not proteins or peptides.**
- ***Bd*** **factors produced after treatment with the chitin synthase inhibitor, nikkomycin Z, have reduced activity, suggesting that they may be cell-wall components.**


Agalychnis callidryas

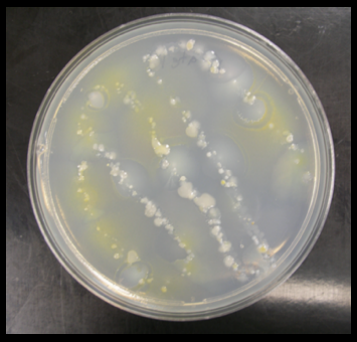
Summary of immune evasion by Bd

- **Enriched cell-wall preparations alone inhibit T cells**
- **Inhibitory activity correlates with carbohydrate content.**
- ***Bd*** **also releases small metabolites, MTA and kynurenine, which may also induce T-regulatory cells or otherwise inhibit lymphocytes.**


Dendrosophus ebraccatus

Role of Skin Microbiota in Protection from Bd

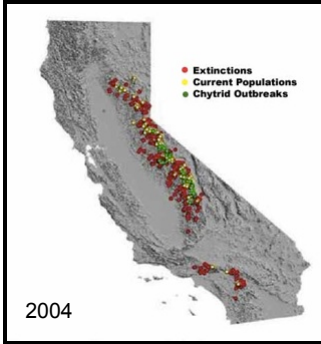
- Amphibian skin hosts a rich array of skin microbes
- Many can be cultured on simple media (R2A) agar



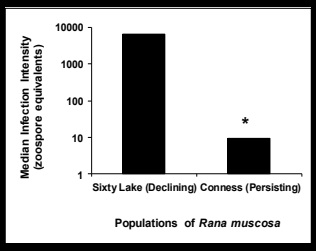
Role of Skin Microbiota in Protection from Bd

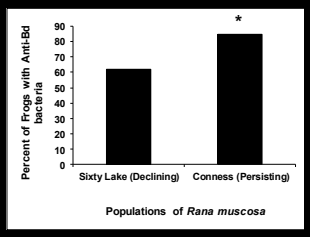
- Amphibian skin hosts a rich array of skin microbes
- Many species can inhibit growth of *Bd*

Role of Skin Microbiota in Protection from Bd



- Microbial species capable of inhibiting *Bd* are more prevalent in *Bd*-endemic populations than naive and declining populations of mountain yellow legged frogs.





Doug Woodhams

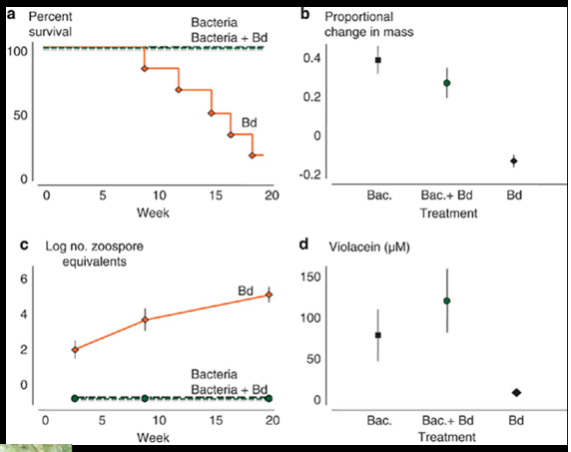
Rana muscosa

Woodhams et al., 2007. Biol. Conserv. 138: 390-398.

With Cherie Briggs, Vance Vredenburg, and Reid Harris

Role of Skin Microbiota in Protection from Bd

- One naturally occurring bacterial species, *Janthinobacterium lividum,* produces an antifungal metabolite called violacein.
- Addition of bacteria to *R. muscosa* juveniles protected from *Bd* infection.

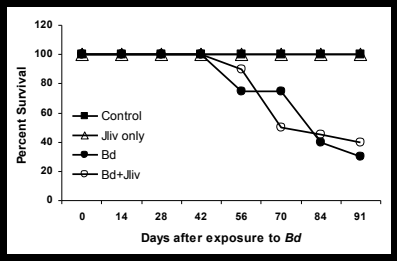


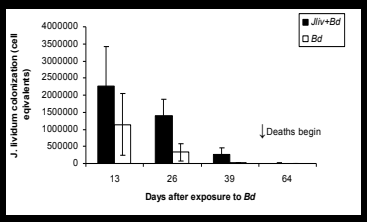
Rana muscosa

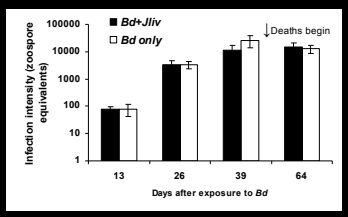
Harris R et al. 2009. ISME J. 3: 818–824

Probiotics or Bioaugmentation

- When *J. lividum* was used to try to protect Panama golden frogs, it did not persist and they were not protected.

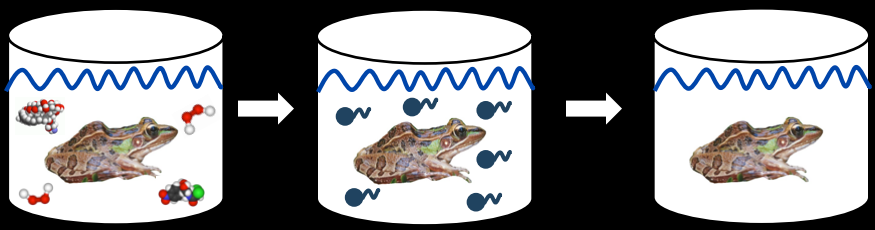






Becker M. et al. 2011. EcoHealth 8:501-506

Does the skin microbiome protect juvenile leopard frogs from infection by Bd?



Use broad spectrum antibiotics to reduce natural bacterial levels.

Expose experimental frogs to *Bd* zoospores* for 24 hours.

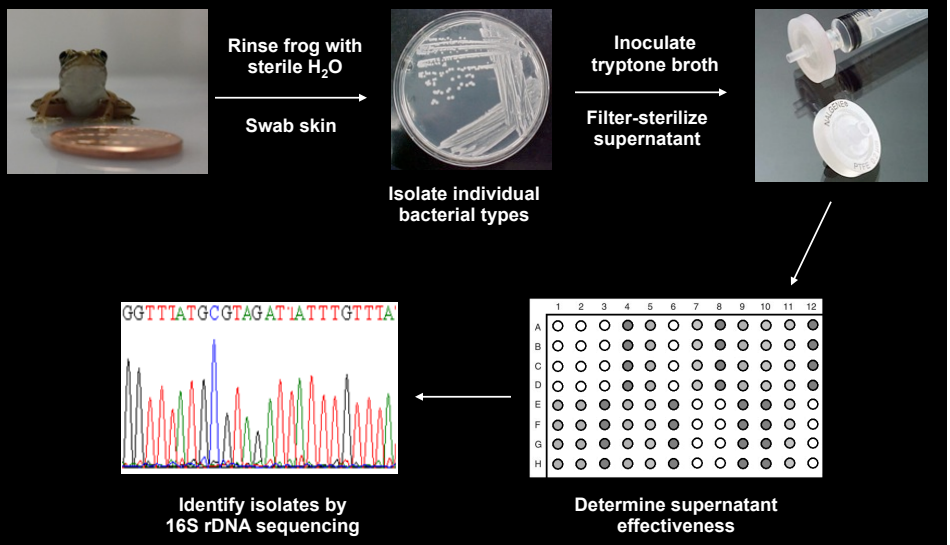
Monitor frog survival, weight loss, and zoospore loads.

*Virulent "Section Line" isolate from J. Piovia Scott and Janet Foley

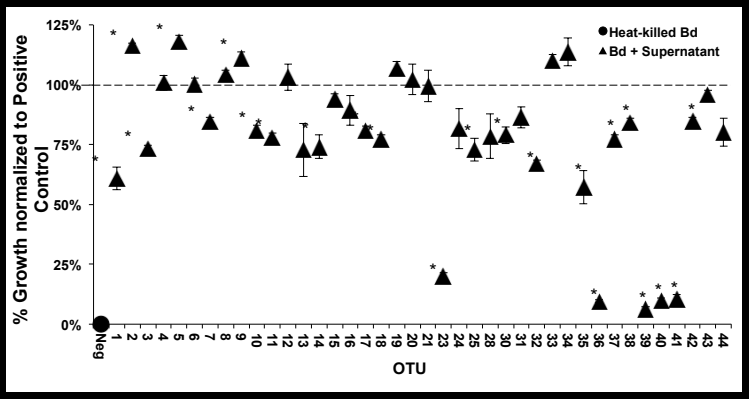
R. sphenocephala metamorphs

- Raised from eggs in mesocosms by Shane Hanlon (grad student with Matt Parris), University of Memphis to generate a naïve population.
- Mesocosms had pond water, soil, leaf litter, algae, and insects from the environment where *R. sphenocephala* eggs were collected.
- Use of mesocosms was important for generating natural complement of skin bacteria.
- Real time PCR confirmed that every metamorph was *Bd*-negative.

What bacteria can be found on the skin of R. sphenocephala metamorphs?



R. sphenocephala bacterial isolates have varying impacts on *B. dendrobatidis* growth



*p ≤ 0.001

With H. Wells and S. Glisson

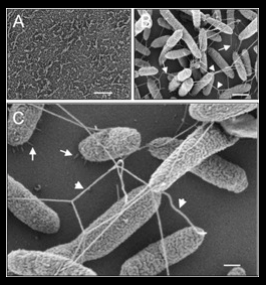
Strongly Inhibitory Isolates

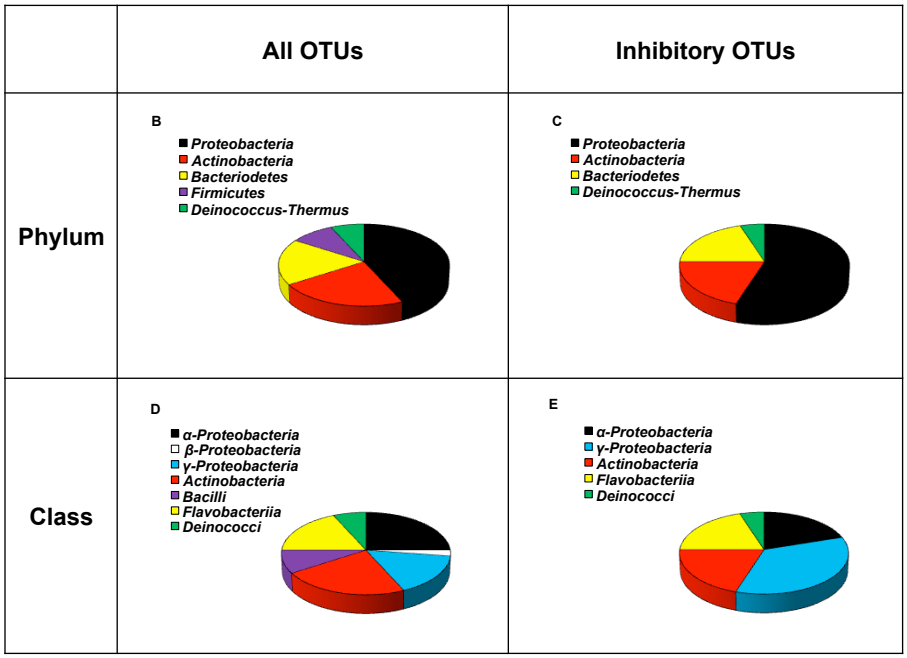
Identity	% Inhibition of *Bd*
Pseudomonas geniculata	93.6
Enterobacter aerogenes	90.4
Pseudomonas hibiscicola	90.3
Stenotrophomonas maltophilia	89.6
Brevundimonas nasdae	79.9

Holden W. et al. 2015. *Biol. Conserv.* 187: 91-102

Protective skin bacteria

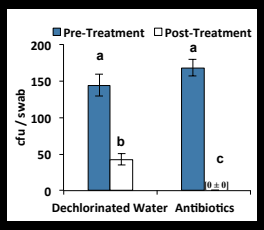
- *Stenotrophomonas maltophilia* has also been identified from skin of amphibians from Tennessee, and California in the USA, Colombia , Panama, and Australia.
- In *Hyalinobatrachium colymbiphyllum* of Panama, this bacterial species was found on the skin of the attending male parent and was also found on developing eggs, suggesting possible transfer to protect the developing offspring.
- Given that it is often cultured from amphibian skin, it may be among a core of potentially beneficial species able to inhibit growth of *Bd*



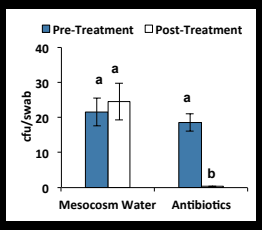


Identification by the team of Valerie McKenzie and Rob Knight and Doug Woodhams

An antibiotic cocktail effectively depletes skin bacteria within 48 hours

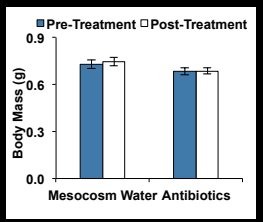


Pilot experiment (N= 5)



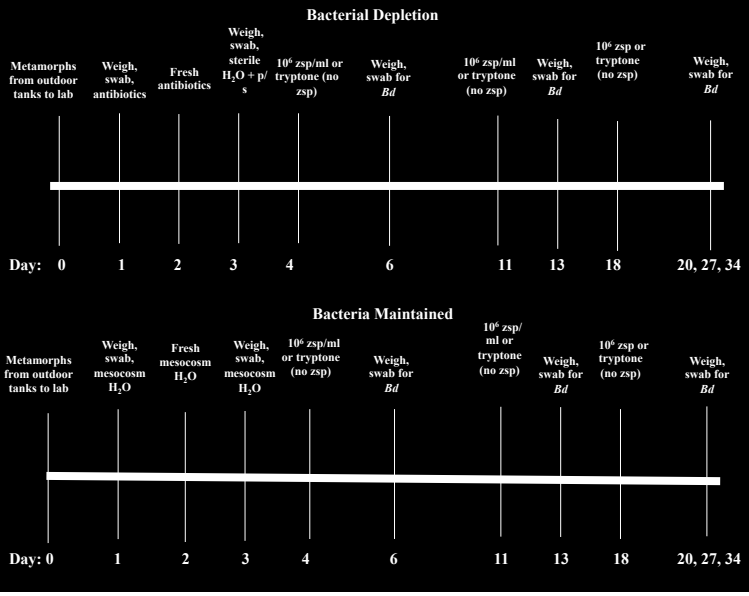
Larger experiment (N = 39)

Antibiotic Cocktail
60 mg/L enrofloxacin
24 mg/L cephalexin
14.5 mg/L sulfamethazine
2.9 mg/L trimethoprim
100 mg/L streptomycin
100,000 I.U./L penicillin

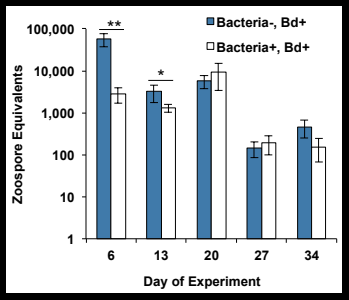


Holden W. et al. 2015. *Biol. Conserv.* 187: 91-102

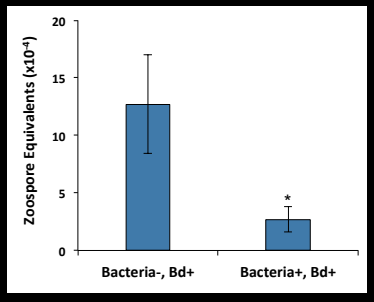
Bacterial depletion experiment



Bacterial reduction results in increased pathogen burden in the first two weeks



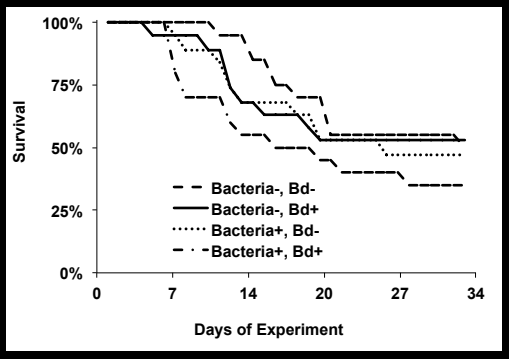
Bd on skin over time with or without bacteria present



Infection load at time of death

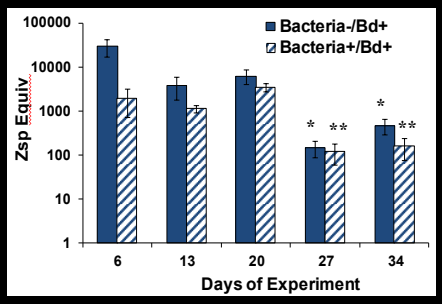
Holden W. et al. 2015. *Biol. Conserv.* 187: 91-102

In spite of the early protection due to presence of natural bacteria, survival was not improved



Holden W. et al. 2015. *Biol. Conserv.* 187: 91-102

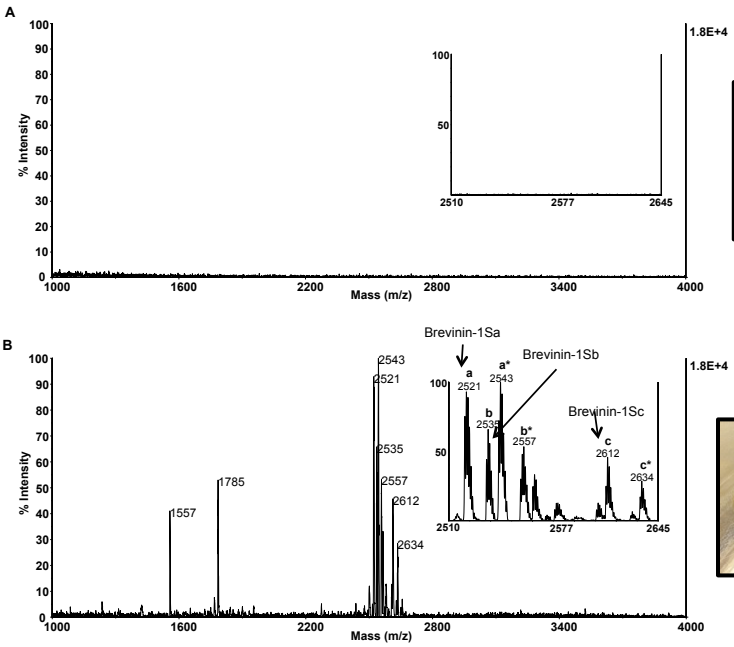
Reduced Bd burdens in longer term survivors suggested development of some protection



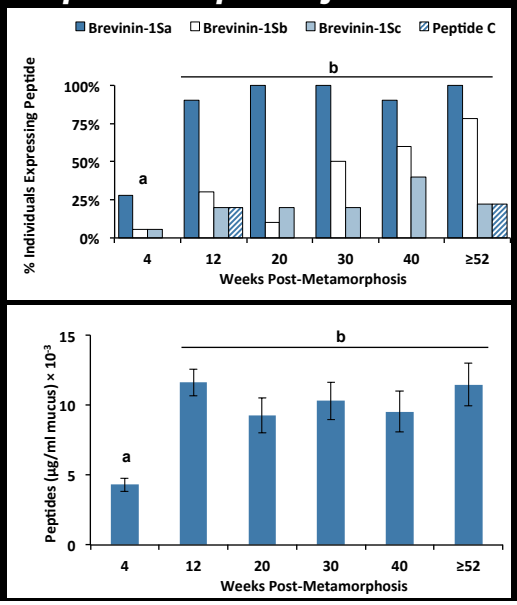
- Among *Bd*-exposed frogs, seven with bacteria maintained and nine with bacteria reduced survived the thirty-five day experiment.
- In these survivors, we examined the *Bd* burden at days 27 and 34 following three exposures at days 4, 11, and 18.
- In comparison with the *Bd* burdens at day 20, *Bd* levels were significantly reduced one and two weeks after the final exposure (days 27 and 34) (repeated measures ANOVA, Tukey post-hoc test).
- Suggests development of resistance.

Holden W. et al. 2015. *Biol. Conserv.* 187: 91-102

At one week-post-metamorphosis, R. sphenocephala do not secrete known AMPs.



Delayed development of AMPs in R. sphenocephala juveniles



Holden W. et al. 2015. *Dev. Comp. Immunol.* 48: 65-75.

Summary of Bacterial Reduction Experiments

- Using the southern leopard frog, *Rana sphenocephala*, we demonstrated that the skin harbors multiple bacterial species capable of inhibiting *Bd* growth *in vitro*.
- Reduction of bacteria on post-metamorphic juvenile skin using a potent antibiotic cocktail resulted in increased *Bd* pathogen burden.

Summary of Bacterial Reduction Experiments

- Despite the beneficial effects of natural bacteria, overall survival against *Bd* was not improved
- In the few juveniles that survived to the end of the experiment, *Bd* burdens (after 3 exposures) were reduced suggesting development of immunity.

Summary of Bacterial Reduction Experiments

- In metamorphic juveniles rapidly developing in mesocosms, an innate bacteria-mediated skin defense may provide some protection at this critical period of development when AMPs and antibody responses are slowly emerging.

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- **Andrea Shiakolas**

2014

2015

Doug Woodhams

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